PULSE ELECTRIC FIELD EXPOSURE EFFECT ON MORPHOLOGICAL PROPERTIES OF HELA CELLS

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ABSTRACT

This thesis is concerned with the investigation of pulsed electric field (PEF) towards biological cells. Biological cells selected in this study are HeLa (cervical cancer) cells. There are two parts of the study, which was involving modeling methods and experimental setup. Modeling method used involves analytical (MATLAB) and numerical (CST®EMS) methods. Both of these methods are to prove the existence of the effect on transmembrane potential changes when subjected exposed to PEF strength. This result can be seen clearly when both method showed the existence of changes effects on transmembrane potential. Therefore, this study continues by identifying an appropriate experimental setup. Experimental setup involves four important parts, the first part is the source of square wave PEF (ECM®830) that can generate until 3kV field strength. Followed by modified EC magnetic chamber with incubator system that has been used in order to exposed HeLa cells to PEF. At the same time this system is coupled with Nikon inverted microscope (Ti-series) for subsequent visualization techniques, image and video. In the early stage, experimental setup was tested by monitoring the proliferation rate of HeLa cells within 0 to 48 hours. Then HeLa cells were tested to look at the swelling effect via PEF exposure. After that, we continued to identify the optimum PEF parameters for reversible condition on HeLa cell. As a result HeLa cells gives a good response at 2.7kV field strength, 30μs pulse length with single pulse. Further study showed that two or more adjacent HeLa cells merge together due to increased cell membrane permeability (electrofusion). This discovery triggered an idea to look at the PEF effect on wound healing process. An artificial wound site were investigated with and without PEF exposure. The finding shows PEF exposed wound area took 3 hours to completely heal while the untreated area took 10 hours. This prove a novel technique (electrical based novel treatment) which could be an alternative to drug usage for wound healing process. Overall, the findings achieved in this study could lead us onto a drug free wound healing method.

ABSTRAK

Tesis ini menjurus kepada penyiasatan medan elektrik denyut (PEF) terhadap sel biologi. Sel biologi yang dipilih dalam kajian ini adalah sel HeLa (kanser servik). Kajian ini terbahagi kepada dua bentuk iaitu kaedah permodelan dan eksperimen. Kaedah permodelan yang terlibat adalah kaedah beranalisis (MATLAB) dan berangka (CST®EMS). Kedua-dua kaedah ini adalah untuk membuktikan wujudnya perubahan upaya terhadap sel transmembran apabila didedahkan kepada kekuatan PEF. Keputusannya, secara jelas menunjukkan bahawa wujudnya perubahan upaya terhadap sel transmembran. Sehubungan dengan itu, kajian ini diteruskan mengenalpasti persediaan eksperimen yang sesuai. Eksperimen ini melibatkan empat bahagian yang utama, bahagian pertama adalah sumber gelombang segiempat medan elektrik denyut (ECM®830) yang boleh menghasilkan kekuatan medan sehingga 3kV. Diikuti dengan pengubahsuaian kebuk magnet EC dengan sistem pengeraman bagi kegunaan pendedahan PEF terhadap sel HeLa. Pada masa yang sama sistem tersebut digandingkan dengan mikroskop (Ti-series) tersongsang Nikon untuk teknik gambaran imej dan video. Pada peringkat awal, persediaan eksperimen ini diuji dengan pengawasan kadar pertumbuhan sel HeLa antara 0 hingga 48 jam. Kemudian sel HeLa diuji untuk melihat kesan pengembangan apabila PEF dikenakan. Seterusnya mengenalpasti parameter optimum PEF untuk keadaan boleh balik sel HeLa pada kekuatan medan 2.7kV, 30μs panjang denyut tunggal. Kemudian, kajian diteruskan dengan melihat kenaikan kebolehtelapan sel membran (elektro-pelakuran) terhadap PEF menyebabkan dua atau lebih sel HeLa yang bersebelahan bergabung menjadikannya bentuk sel yang besar. Penemuan ini menjadi pemicu kepada idea untuk melihat kesan PEF terhadap proses penyembuhan luka. Sehubungan dengan itu, kajian terhadap luka buatan dibangunkan mnggunakan sel HeLa di mana, sampel sel tersebut dibahagikan kepada dua bahagian iaitu didedahkan dan tidak didedahkan dengan PEF. Keputusannya memperlihatkan hanya 3 jam diambil untuk penyembuhan luka jika dikenakan PEF berbanding 10 jam dalam keadaan biasa. Kesimpulannya, teknik novel (rawatan berasaskan elektrik) boleh menjadi alternatif kepada penggunaan dadah untuk proses penyembuhan luka seterusnya pencetus kepada kaedah yang bebas dadah dalam penyembuhan luka.

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LIST OF SYMBOLS AND ABBREVIATIONS

CST® EMS	Computer Simulation Technology®Electromagnetic Studio
ECM®830	Square Wave Pulse Generator
EP	Electroporation
FIT	Finite Integral Technique
PEF	Pulse Electric Field
TA	Threshold Area
HELA	Cervical Cancer Cell

LIST OF PUBLICATIONS, RESEARCH GRANT AWARD AND RESEARCH COMPETITION

The followings are the list of publications relevant to this thesis.

Book Chapter:

1. **Adon, M Nazib**, M Noh Dalimin, N. M. Kassim, and M.M A.Jamil. 2011. Microdosimetry Modeling Technique for Spherical Cell." In *5th Kuala Lumpur International Conference on Biomedical Engineering 2011: BIOMED 2011, 20-23 June 2011, Kuala Lumpur, Malaysia*: Springer. 447.

Conference Proceedings:

2. **Adon, M. N**., M. Noh Dalimin, M. M. A. Jamil, and N. M. Kassim. 2012. "Development of high voltage pulse inducement method for biological cell." In *Biomedical Engineering (ICoBE), 2012 International Conference on*. 501-503.

3. **Adon, M. N**., M. Noh Dalimin, M. M. A. Jamil, N. M. Kassim, and S. Hamdan. 2012. "Study of effect of microsecond pulsed electric fields on threshold area of HeLa cells." In *Biomedical Engineering and Sciences (IECBES), 2012 IEEE EMBS Conference on*. 484-486.

4. **Adon, M. N.**, M. Noh Dalimin, M. M. A. Jamil, N. M. Kassim, and S. Hamdan. 2013. "Electrofusion effect of induced transmembrane potential under a live - Cell microscopy system." In *Biomedical Engineering International Conference (BMEiCON), 2013 6th*. 1-3.

5. Zaltum, Milad, A Mohamed, **Adon, M.N.**, M. M. A. Jamil. 2013. "Electroporation effect on growth of HeLa cells." In *Biomedical Engineering International Conference (BMEiCON), 2013 6th*: IEEE. 1-4.

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CHAPTER 1

INTRODUCTION

Human cell biology system is too complex to understand. Most scientists have been doing a lot of research intensively to understand the effects of stimulus that occurs between the human cell biological system against external factors, including the effects of electric fields applied in different intensities and durations. In this chapter we will emphasize of discovery the new phenomenon electric field impact on human cell biology performed.

1.1. Background of Research

The earliest written record on biological cell effects of electric fields have been reported over the past fourth decades. Neumann firstly reported in 1972 permeability changes induced by pulsed electric field in cell membrane (Neuman. and Rosenheck. 1972). Zimmermann explained the permeability changes as a pore formation of membranes due to its electrical breakdown, such as electroporation (Zimmerman., Pilwat. *et al.* 1974). In other words, electroporation is a technique in which electric pulses are used to create transient pores in the cell membrane used for the delivery of biologically active molecules into cells (Smith, Neu *et al.* 2004).

Most studies relating electroporation behavior are involving in-vitro techniques which engage various processes of culturing human cells. Among the cells used in this process include melanoma cells (Petrishia and Sasikala 2014), J3T

(brain tumors) cells (Neal Ii, Rossmeisl Jr *et al.* 2014), Chinese hamster ovary (CHO-K1) cells (Thompson, Roth *et al.* 2014), and HeLa (cervical cancer) cells (Zhang, Xiong *et al.* 2013). Therefore there are a variety of cells that can be used and categorized it as different cell shape, structure and content. Thus cell selection is very significant in the process of electroporation which every type of cell has a different consequence towards electric field intensities and duration.

Since the structures of cells are too complex, various modeling methods are introduced to represent the cells structure with simplified model have been used to studying the cells, such as circuit model (Roy and Barman 2014), parallel plate models (Hsiao, Choi *et al.* 2013), and the layered model (Mesin 2013). In essence, it is very useful to examine transmembrane potential, pore formation, reversible and irreversible electroporation process in greater depth and detail .

However, to prove the validity of the model, real time experimental setup must be develop to verify the existence of electric field effects on cells. Previous study showed there are two areas of study involving the experimental analysis of electroporation process namely bioscience and bioengineering . Most biotechnology application research involves electroporation to allowing chemicals, drugs or DNA to be introduced into the cell which is relevant for the purposes of medical applications and genetic studies (Lindstrom, Brewer *et al.* 2014). In contrast, bioengineering research concentrate on transient aqueous pores form in lipid bilayers, that is fundamental mechanism of large electric fields may alter physiological and morphological on cell (Thompson, Payne *et al.* 2011). Therefore, the requirement of high voltage intensity with multiple pulses are indispensable in realizing each experiment carried out effectively.

Recently, the unique pulsed electric field effects against biological cell, has opened a new gateway to tumor treatment and become a research focus in the area of bioelectromagnetics (Ferreira, Saga *et al.* 2013; Gehl, Linnert *et al.* 2013; Mali, Jarm *et al.* 2013). There are numerous experimental research show that pulsed electric field with different parameters can cause different bioelectric effects. Weaver et al. found that in response to microsecond pulsed electric field (typical parameters: 1

kV/cm, 100μs), many reversible aqueous channels, which are often called pores (radius ≈20-110 nm), appear at the cell membrane, while there is no obvious effect on the transmembrane organelles. This physical procedure is termed electroporation, which can make cell membrane more permeable to drug molecule. The following technique has been successfully applied to tumor treatment (Weaver 2000; Weaver 2003).

However, most of the electroporation experiments using a cuvette to hold samples with a variety of electrode gap sizes. Three electrode gap sizes are available, 1mm for bacteria and yeast, 2mm for all cell types and 4mm for mammalian cells. The cuvette are molded with embedded polished aluminum electrodes, and gamma irradiated for guaranteed sterility. Nonetheless, this cuvette system cannot be integrated with real time visualization using high resolution microscopy.

Therefore, the experimental setup with environment controlled system must be identified to make all observation during pulse electric field induced can be recorded in real time visualization with high resolution microscopy system. As a result it could be concluded that pulsed electric field with different duration and intensity can cause various biomedical effects such as morphological changes on cells, which suggests a various types of external pulsed electric field and biological cell. Since then there have been a number of studies on the mechanism of electroporation and its applications to gene transfection (Dower, Miller *et al.* 1988), cell fusion (Zimmermann, Friedrich *et al.* 2000) and medical treatments (Dev, Rabussay *et al.* 2000).

1.1.1. Introduction to Electroporation

The developmental of micro and molecular biology, chemical and biological techniques have been developed to transfer the selected material through the cellular membrane (Ausubel, Brent *et al.* 2002). The capabilities of implemented transmembrane transport of materials is crucial to many areas of research. Most studies involve the transport of macromolecules such as DNA, RNA, antibodies, chemical drugs, metabolites, molecular probes and multiple vesicles.

Research related to electroporation (EP) has attracted more scholarly among cell biologists and biophysicists, is that high-voltage electric pulses can generate fusion cells. Giant cells viability was first obtained by Neumann et al. (Neumann, Gerisch *et al.* 1980) with a simple electro-pulsing of a suspension of cells. Later, it was proposed to use the phenomenon of dielectrophoresis (Pohl 1978), to obtain close contact between cells (Scheurich, Zimmermann *et al.* 1981). Dielectrophoresis is the movement of a relatively nonconducting particles or charged (cells) in a non-uniform AC electric fields (Pohl 1978). If several particles are present, the appropriate particle size, particle density, electric field magnitude and frequency can cause the cells to aggregate in long chains (pearl chain) in an alternating electric field depending on their effective polarisability (Zimmermann 1982).

Neumann et al. (Neumann, Schaefer-Ridder *et al.* 1982) have reported a method of transfection of foreign genes into eukaryotic cells by electroporation method. Transfection involves opening transient pores in the plasma membrane of cells, to permit the taking of genetic material or proteins such as antibodies. It was also reported that the transfected genes expressed in the host cells (Neumann, Schaefer-Ridder *et al.* 1982). Following the discovery, electroporation has become accepted as an effective technique for the introduction of foreign DNA into cells of any origin (Potter 1988; Neumann 1989).

Further techniques have been developed in various fields including biochemistry, genetics, medicine, pharmacology, immunology, microbiology and toxicology. Utilities in vivo electroporation for the entry of molecules has been demonstrated by the increasing number of new applications have been developed each year (Mir 2001).

Experiments in 1988 on human skin fibroblast showed that highly efficient transient chloramphenicol acetyltransferase expression was shown after transfection with plasmid (Fountain, Lockwood *et al.* 1988). The ability to easily transfect these cells with exogenous DNA may have important applications in the study of human genetic diseases and cancer. Further research has shown that the electroporation of

the skin could be used to enhance transdermal drug delivery (Prausnitz, Bose *et al.* 1993; Prausnitz, Pliquett *et al.* 1994) .

In 1993 it was reported that upon application of electric fields pulses on a suspension of cells in the presence of a selected membrane protein, implantation of the protein in the cell plasma membrane was possible (Nicolau, Mouneimne *et al.* 1993). This phenomenon is called electroinsertion. Later, electroporation of excitable membranes was observed (O'Neill and Tung 1991). Electrically induced membrane breakdown of isolated cardiomyocytes cells was reported the morphological evidence for the presence of RhoA protein (Wang, Tsai *et al.* 1997).

A method of electroporation has been applied in vivo to introduce anticancer drugs to tumorous tissue (electrochemotheraphy) in order to obtain therapeutic effects by using short, intense electric pulses that surpass the capacitance of the cell membrane (Gothelf, Mir *et al.* 2003). The main factors that play a crucial role in obtaining high responses of the treatment are the drug used in the treatment and the appropriate electric pulses delivered to the tumor.

Electroporation has been focuses on the selective sterilization of fermented foods by intense pulsed electric field. Since intense electric field can make pores penetrating a membrane of a cell, the principle of the sterilization by intense electric field is irreversible electroporation that releases the contents through the pores of the cell membrane of microorganisms, it is simpler and more efficient than rival chemical and biological processes (Manabe, Nakagawa *et al.* 2011; Saito, Minamitani *et al.* 2013; Manabe, Maetani *et al.* 2014).

However, the mechanism of electroporation is still not fully understood and there are aspects of the process that has yet to be reviewed to see its effectiveness. Which is related to the parameters of electric pulses optimal for specific cell to the desired application (Hibino, Itoh *et al.* 1993; Jaroszeski, Heller *et al.* 2000; Satkauskas and Saulis 2004). The scope of this thesis is to address some of these problems in an attempt to increase understanding and efficiency through optimization of parameters for electroporation.

1.2. Problem Formulation

However, the inducement process of external pulse electric field towards cells is still not completely understood. Therefore, there is need for supplementing experimental knowledge with theoretical models (Valič, Golzio *et al.* 2003; Krassowska and Filev 2007; Chengxiang, Chenguo *et al.* 2011; Moen, Roth *et al.* 2013). This is due to the small size and thickness of cells and their thin membranes resulting in great difficulty for experimental investigation and applicable of numerical technique.

In this thesis, analytical and quasistatic approximation techniques have been used to investigate the interaction of pulsed electric field with biological cells. The outcome of these assessments will guide the experimental evaluations of pulsed electric field influences on biological materials at cellular level. More specifically, the studies presented in this thesis will clarify; (i) the field intensity and potential difference built on cell membrane; (ii) development of experimental setup for real time study of morphological features on cell membrane under pulsed electric field exposure combined with high resolution microscopy imaging features.

1.3. Aims and Objectives

The primary aim of this study is to examine the phenomenon of biological effect on electric field pulses towards HeLa cell. To enable this, the thesis is presented in two main parts:

Part I: In which the work will firstly investigate the association between the effects of the electric field strength that produces towards spherical cell model for transmembrane potential, where the analytical and numerical models developed for spherical cell to prove this theory. This will then lead towards development of controlled electroporation technique for HeLa cells, to improve this process using live imaging techniques in real time (Chapter 3, 4 and 5).

Part II: In which the newly established electroporation technique will be investigated to examine optimization of pulsed electrical field (PEF) and the number of pulse towards morphological changes on HeLa cell. This will then lead towards

phenomena of electrofusion and thus, discovery of wound healing process assisted by electric field excitation (Chapter 6 and 7).

1.4. Scope of Research

In order to achieve the objective of this research, following scope of work have been identified which comprises of:

1. Modeling and simulation of single and multi layer for spherical cell shape based on quasistatic approximation approach by analytical and numerical method.
2. Preparing a complete HeLa cell culture protocol on 25cm^2 flask in medical instrumentation laboratory.
3. Develop a real time visualization setup for pulse electric field effect exposure on HeLa cells.
4. Preparing a complete HeLa cell culture protocol on CMB and EC magnetic chamber for control environment and real time visualization.
5. Characterize an optimization of high voltage pulse electric filed exposure for electroporation process.
6. Real time morphological observation of multicellular behavior during PEF exposure for qualitative analysis.

1.5. Overview of Thesis

This thesis has been organized into nine chapter as follows:

Chapter 1 Introduction: A brief review of the topic and a general introduction will be focused in this chapter. The fascinating aspects on relationship between biological cells and pulse electric field studies will further investigated. According to previous

studies, the problem formulation will be identified, therefore aims and objectives will be established in this chapter.

Chapter 2 Electroporation In Cell Biology: This chapter will review on theory of electroporation that involved biological cell physics structure, modeled and medical applications for extended understanding in this studies.

Chapter 3 Quasistatic Technique on Cellular Scale: This chapter presents an analytical (MATLAB) and numerical (CST®EMS) evaluation of spherical cell model, the electric field distribution on cell membrane are evaluated with presence of effects transmembrane potential.

Chapter 4 Development of Controlled Electroporation Technique for HeLa Cells: This chapter will focusing on development of environmental controlled experimental setup for electroporation process specifically for HeLa cells. Moreover, cultured HeLa cells technique will be performed in this studied to investigate on proliferation rate (48 hours) on 25cm^2 flask.

Chapter 5 A Preliminary Effect of Electroporation Process on HeLa Cells: This chapter investigated the preliminary effects on morphological changes during inducement of pulse electric field towards HeLa cells. The morphological changes will be observed by using real time high resolution microscopy.

Chapter 6 Optimization of EP Technique on Cellular Scale: This chapter studied on optimization of electric field intensity using square wave pulse generator (ECM®830) to get reversible electroporation behavior as recorded via time lapse application.

Chapter 7 Pulse Electric Effect on HeLa Cells: This chapter will observed and demonstrated on plasma membranes at points of contact between adjacent Hela cells to look into morphological features during exposure of optimum field strength performed. Furthermore this chapter will be investigated on wound healing process when HeLa cells were exposed to pulse electric field and the wound closure will be observed with real time visualization.

Chapter 8 Overall Conclusion: This chapter will summarize the conclusions drawn from the experimental results acquired in this thesis and give recommendation for potential future work.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

This chapter will emphasize to the theory of electroporation, including a theoretical on cell structure component that govern the manipulation of cells by electric field and also on spherical cell modeling which will be employed in this research work. As already known electroporation refers to the ability of high magnitude electric fields to alter the permeability of the cell membranes. A change in the permeability of the cell membrane leads to the existence of microscopic 'pores'. This pores is usually called ‘electropores,’ or also known as electropermeabilization.

2.2. Response of Cells to Electric Field

Electroporation phenomenon was first described by Neumann and Rosenheck (Neumann and Rosenheck 1972). Followed by gene transfer via electroporation method been achieved a decade later (Neumann, Schaefer-Ridder *et al.* 1982). This development resulted in this technique is now widely used in laboratories around the world for use in the clinical setting (David et al., 2008)(Daud, DeConti *et al.* 2008) and increasingly more attention in recent years as a method for introducing foreign genes into cells in vivo (Muramatsu, Arakawa *et al.* 2001). Electroporation has been used in the past decade as well as to improve cancer drug delivery to the cells. Pre-clinical investigation in the late 1980s (Mir, Orlowski *et al.* 1991; Mir, Orlowski *et al.* 1995), followed by the first clinical trial in 1991 (Mir, Orlowski *et al.* 1991).

Currently numerous research on biotechnology applications require the transport of macromolecules such as antibodies, genes, drugs into the host cell. Each selected applications to be able to do the transfer process given is based on

effectiveness, ease of use and side effects. Therefore, the method that is both versatile and efficient are being sought and investigated. An important factor in all these applications, the viability of cells needs to be conserved. However, in some applications of biotechnology, such as sterilization of liquid food or water treatment, electroporation is used as a method to kill cells efficiently.

Electroporation is a process of membrane phenomenon which involves fundamental behavior of cell and artificial bilayer membranes, and increasingly attracts consideration for applications in biology, biotechnology and medicine. Essential features of electroporation include application of short electrical pulses, charging of lipid bilayer membranes, rapid localized structural rearrangements within the membrane, transitions to water filled membrane structures, which make a hole in the membrane ("aqueous pathways" or "pores") and tremendous increase in ionic and molecular transport. On the other hand polarization is one of the basic mechanisms of interactions of membranes with electric fields, leading to electroporation and related phenomenon of dielectrophoresis and electrofusion. Understanding the underlying interaction mechanisms caused by pulsed electric field exposure is necessary for assessing the possible impact on biological cell particularly to cell membrane.

2.2.1. Parameter Influencing Electroporation

The most important parameters for effective electroporation are the electric field strength and the duration of the field is applied (pulse length). A large variety of other parameters can influence the efficiency of electroporation, such as the shape of the electrical pulse, polarity, number of interval between pulses, size of target cells and thermal conditions during and after the pulses. The uptake of molecules also depends on their molecular sizes, charges, and other physical and chemical properties. The relationship between the two basic parameters, field strength and pulse length, is shown in Figure 2.1.

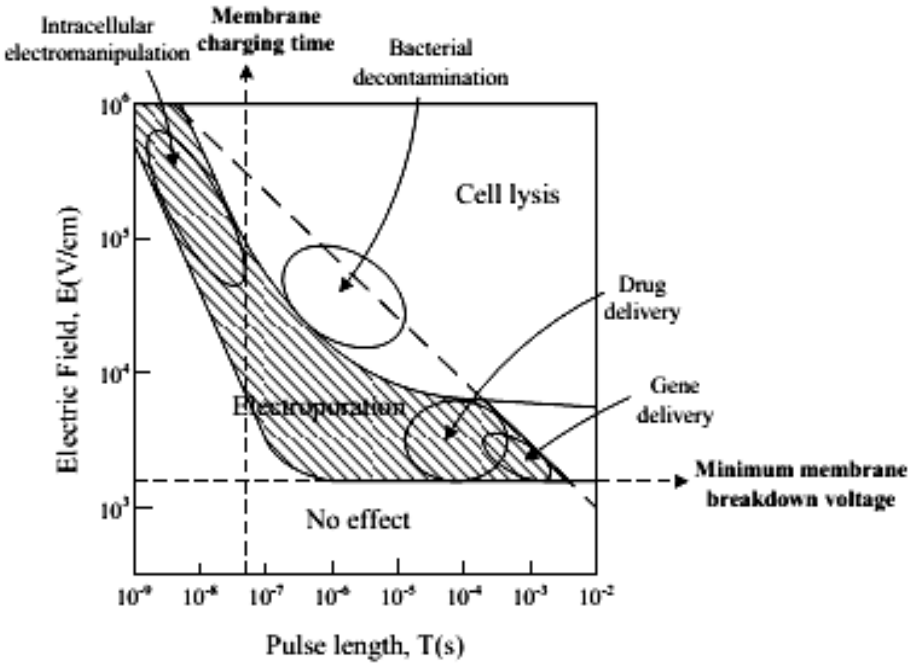

Figure 2.1: Parameter range for bioelectric applications (Electric field *E* – Pulse length *T*) (Wang 2009)

As shown in Figure 2.1, in the range of small electric field - pulse duration (E-T), the poration will not happen. With increasing field intensity or exposure duration, it approaches the range where more obvious effects are expected, even if temperature changes are still tolerable. When E-T increases to a vital dosage, the cells under exposure could be killed, that is the cell lysis region.

Besides different E-T products, different applications require working in different regions of this E-T map. For medical applications, the range of long pulses and low-electric fields on the right of Figure 2.1is the preferred range of operation. Particularly, gene transfection occurs with pulsed power parameters on the far right, pulse durations in the microsecond range(typically 20ms), and electric field amplitudes in the order of 100 V/cm. Electrochemotheraphy (drug delivery) requires higher electric fields (kilovolts per centimeter) and shorter pulses (>10μs). Bacterial decontamination requires pulse durations in near microsecond range, operating at electric fields from 10 to more than 100 kV/cm. When it moves to very short pulses and very high-electric fields in the left-hand corner of this diagram, a completely different range of applications appears. Because the pulse duration is smaller than the

membrane charging time, the subcellular effects instead of plasma membrane electroporation contribute to the intracellular electromanipulation.

2.2.2. Formation of Pores

The transmembrane potential is valid only until pores are formed. Once enough pores are formed, the membrane conductivity changes and Schwan's equation is not valid any more. This phenomenon of electroporation has often been referred to as `electrical breakdown' or electropermeabilization. A few well observed and documented characteristics of the cell membrane electroporation can be summarized as follows:

1. The transmembrane potential must exceed a certain threshold value $V_m(cr)$ for electroporation to occur (Hibino et al., 1991; Kinosita et al.,1992).
2. It is thought, most probably, that it is the lipid part of the biological membrane which is transiently permeabilized by an electroporation pulse (Chernomordik et al., 1987).
3. Electropermeabilization of cells can be asymmetrical: pore populations in two hemispheres may differ in the size and (or) number of pores (Kinosita et al., 1992).
4. The change of the membrane permeability caused by the pore formation can be fully reversed. When the pulse parameters, number of pulses and the medium properties are properly chosen, electropores have a finite lifetime (Swezey and Epel, 1989; Kinosita and Tsong, 1977a,b; Saulis and ^ Satkauskas, 1977; Saulis et al., 1991).
5. The increased permeability can be sufficient enough to allow ions and small molecules as well as macromolecules to enter or leave the cell (Kinosita and Tsong, 1977b; Liang et al., 1988; Graziadei et al., 1991; Sheng et al., 1995; Swezey and Epel, 1989; Yumura et al., 1995).
6. The uptake through pores is greater in a solution of low ionic strength (Kinosita and Tsong, 1977a; Rols and Teissie, 1989; Teissie and Tsong, 1981).
7. Permeability is bidirectional, that is, intracellular compounds (e.g., ions,glycine, ATP, proteins) can leak from electroporated cells (Moser et al.,1995; Neumann

and Rosenheck, 1972; Schwister and Deuticke, 1985), while foreign substances can enter the cell (Kinosita and Tsong, 1977a,b; Swezey and Epel, 1989; Zimmermann et al., 1980).

8. Phospholipids in the membrane exhibit major structural changes under electroporation conditions (Neumann et al., 1992). There appears to be a rapid transition (within 1 s) from hydrophobic to hydrophilic pores.

This dependence of high voltage pulse induced to transmembrane of cell are researched theoretically and practically in chapters 3 to 8. In early years a basic concept of the transient aqueous pore hypothesis was that they are membrane `defects' or `membrane perforations' (Neumann 1989), that are created with rapidly increasing rate as transmembrane potential V_m is increased. If the magnitude of V_m increases from zero due to the Figure 2.2: Types of electropores: (a) Hydrophobic (nonconduting pore), (b) Hydrophilic pore (conducting pore) applied external electric field, then the additional membrane energy associated with V_m leads to increased pore creation probability. The rate of pore creation increases nonlinearly with larger V_m. A pore population described by a pore density function quickly increases with respect to increasing V_m, and gives the cell membrane rapidly changing electrical conductance thus reducing the rate at which pores can be created.

Figure 2.2: Types of electropores: (a) Hydrophobic (nonconducting pore), (b) Hydrophilic pore (conducting pore).

2.2.3. Reversible and Irreversible Electroporation

Reversible electroporation happen when a cell is exposed to a sufficiently high electric field, its membrane becomes temporarily permeable to molecules that

otherwise cannot pass through it. This process has been used as a tool for introducing foreign substance such as exogenous DNA into cells (gene electrotransfer) (André, Gehl *et al.* 2008) or membrane-impermeant drugs in order to kill cancer cells (electrochemotheraphy) (Gothelf, Mir *et al.* 2003),(Sersa, Miklavcic *et al.* 2008). Otherwise, irreversible electroporation occurs when the electric field applied results in leakage of cellular components, which leads to cell death. The method was used in microbiology in order to kill bacterial cells (microbial deactivation) (Castro, Barbosa Canovas *et al.* 1993),(Ho and Mittal 1996) and in medicine to ablate tissue nonthermally (Edd, Horowitz *et al.* 2006).

2.3. An Experimental Studies of Electroporation

The activity of experimental studies of electroporation is mainly focused on credible experimental observations over theoretical models. For this reason, various methodologies can be applied to investigate the effects of EF exposure on living cells.

As already noted experiments conducted on artificial bilayers, suspensions of vesicles or cells, and tissues have demonstrated that a large force applied externally induced transmembrane potential (V_m) causes an increase in the conductivity of the membrane by five to six orders of magnitude (Abidor, Arakelyan *et al.* 1979; Benz, Beckers *et al.* 1979).

This effect is generally attributed to the creation of pores, which are the aqueous pathways in the lipid bilayer of the membrane, and whose creation and subsequent growth are facilitated by large Vm. This process, called electroporation, can be irreversible, leading to a mechanical rupture of the membrane, (Sano, Arena *et al.* ; Abidor, Arakelyan *et al.* 1979; Diederich, Bähr *et al.* 1998) or reversible, in which case pores reseal and the same membrane can experience multiple episodes of the high conductivity state (Benz, Beckers *et al.* 1979; Chernomordik, Sukharev *et al.* 1983).

Because of great interest in this method, studies use a variety of experimental techniques to provide insight into the processes taking place during electroporation.

Many of the techniques applied are focused on measuring the time course of transmembrane voltage or current (Tsong 1991) through the membrane(Zimmerman., Pilwat. *et al.* 1974; Melikov, Frolov *et al.* 2001), monitoring uptake or leakage of fluorescent molecules (Teissie and Tsong 1981; Tekle, Astumian *et al.* 1994; Gabriel and Teissié 1997; Gabriel and Teissié 1998), the potential transmembrane imaging (Hibino, Shigemori *et al.* 1991; Knisley and Grant 1995; Nikolski and Efimov 2005), measuring the tissue impedance (Ghosh, Keese *et al.* 1993; Huang, Sekhon *et al.* 2003), and observing pores with rapid-freezing electron microscopy (Chang and Reese 1990).

However, electroporation is difficult to observe directly because pores are very small (nanometers) and their creation and growth is very fast (microseconds), so many things that need to be considered in doing the experimental process, although considerable progress has occurred, there are basic aspects of electroporation that have not been thoroughly determinate experimentally(Weaver and Chizmadzhev 1996; Neu and Neu 2009; Yarmush, Golberg *et al.* 2014).

Although the electroporation is currently the subject of increasing study, it is far from being fully understood. There are various methodologies can be applied to investigate the effects of EF exposure on living cells. This thesis will discuss two methods of study which are often discussed in terms of modeling and experimental. Where the basis of the development of this experimental setup will give a clearer picture of the best applications for the EP..

2.3.1. In-Vitro Methods

The most popular technique used by researchers for the early stage of the electroporation experimental will choose in-vitro method. In-vitro method refers to the technique of performing a given experiment in a controlled environment outside of a living organism (Tsuru, Nagata *et al.* 2002; Li 2004; Kim, Cho *et al.* 2008; Hovis, Padmanabhan *et al.* 2010; Wells 2010; Kulbacka, Nowak *et al.* 2011; Wezgowiec, Kotulska *et al.* 2013; Sano, Arena *et al.* 2014). This method introduced

the sample studied, among tissues and cellular-level interactions with the EF exposed under controlled environments.

The main advantage of such studies is that some of the exposure conditions can be easily and precisely controlled (for example, changing exposure duration, background temperature, or exposure field intensity) as a means of determining dose-response relationships, and the effect of applying different threshold levels (Tattersall, Wood *et al.* 1999). These factors are essential to understand the quantitative interaction mechanisms. The disadvantage of in-vitro testing is that the tissues and cells are isolated from the complete complex systems of the body (Yu, Qiao *et al.* 2006). Thus, any effects observed in-vitro needs to be carefully translated back to the whole body system scenario.

2.4. The Biological Cells

The biological cells is the basic structural, functional and biological unit of all known living organism. The human body is composed of trillions of cells (Curtis and Barnes. 1983). The human body system can be divided into three distinct parts, namely the structure of various cell types, tissues and organs as shown in Figure 2.3. These parts consist of an extraordinary complex arrangement in which cells are the basic unit of structure and play a role in all organisms.

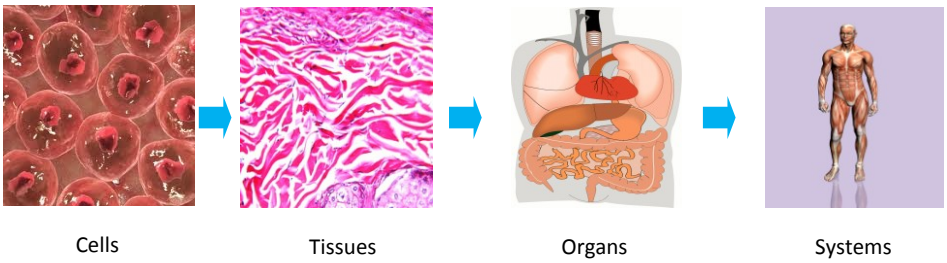

Figure 2.3 : Three distinct parts for the human body system.

Each cell is self-contained and self sustaining nutrients in which able to change and take nutrients into something else that can help in multiplication, expansion, power, or defense. However, each cell is made from the same set of components. The common component of every cell is the cell membrane and the genetic material that determines the specific type of cell. Cell membrane is a physical barrier that separates the cell interior and its environment, controls what moves in and out, and maintains the electrical potential between cell interior and cell exterior. Two different types of the genetic material organization of the cell: the prokaryotic and the eukaryotic cell.

Prokaryotic cells (Figure 2.4) are usually singletons, they have no true nucleus as the DNA is not contained within a membrane or separated from the rest of the cell, but is coiled up in a region of the cytoplasm called nucleoid. The primary example being bacteria and their cells are not as complex as eukaryotic cells.

Prokaryotic cells also have a cell wall which is produced by the cell and resides on the outer surface of the cell membrane. A cell wall is one that worked an outer covering of most cells that protects the bacterial cells and gives it shape.

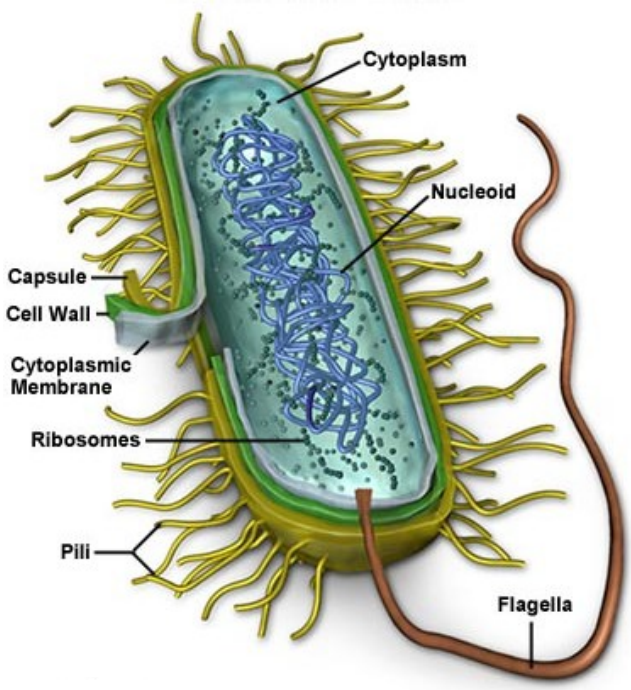

Figure 2.4 Illustration of complex anatomy of a prokaryotic cell structure. Adapted from (http://www.microscopemaster.com/prokaryotes.html/, 2013).

In contrast eukaryotic cells (Figure 2.5) are usually found in multicellular organisms like plants and animals. Eukaryotes (meaning truly nucleus) separate most of their genetic material into a well defined region, called the nucleus, surrounded by a double membrane sack known as the nuclear envelope.

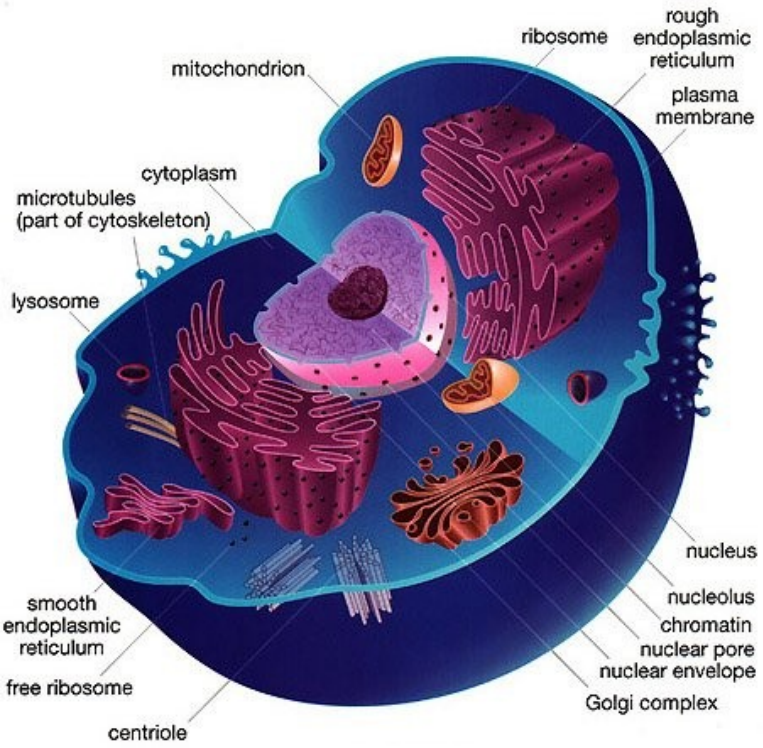

Figure 2.5 Illustration of complex anatomy of an eukaryotes cell structure. Adapted from (http://commons.wikimedia.org /wiki/File:Eukaryotic_Cell_%28animal%29.jpg/, 2013).

Some eukaryotic cells also produce cell walls, but these are quite different to those of the prokaryotic cell. All other components of a cell are inside the cell, and are known as the cytoplasm. The cytoplasm contains all the molecules required for existence, in addition to well defined regions of function such as the organelles (meaning little organs). In order to assist support the cell and maintain its shape, a thin semi-permeable membrane (cell membrane) must be surrounds the cytoplasm of a cell. Animal cells, plant cells, and fungal cells have a cell membranes.

2.4.1. The Cell Membrane

Cell membranes have been widely being discussed in the development of electroporation process. The cell membranes are complex, separates a cells interior from the surroundings, controls what moves in and out, and maintains electric potential of the cell. A membrane is made mostly from a double layer of lipids hydrophobic fatty acid chain molecules (hydrophobic tails) and hydrophilic phosphorus molecules (hydrophilic heads). Hence, the membrane is called a phospholipid bilayer. Lipids are water soluble, oily (greasy) organic substances, and are the most important storage forms of chemical energy in the body (Tobin and Morel 1996). A major component of the cell membrane are polar lipids. The cell membrane is also the basis for the capacitive nature of cells and tissues.

In Figure 2.6, the phospholipid heads cover the two surfaces of the bilayer and the fatty acid tails constitute the interior of the bilayer (Tsong 1991; Casey 1995; Lipowsky 1995). The membrane embedded proteins, and sodium ionic channels are shown. The proteins are mostly involved with selective molecule transport across the membrane.

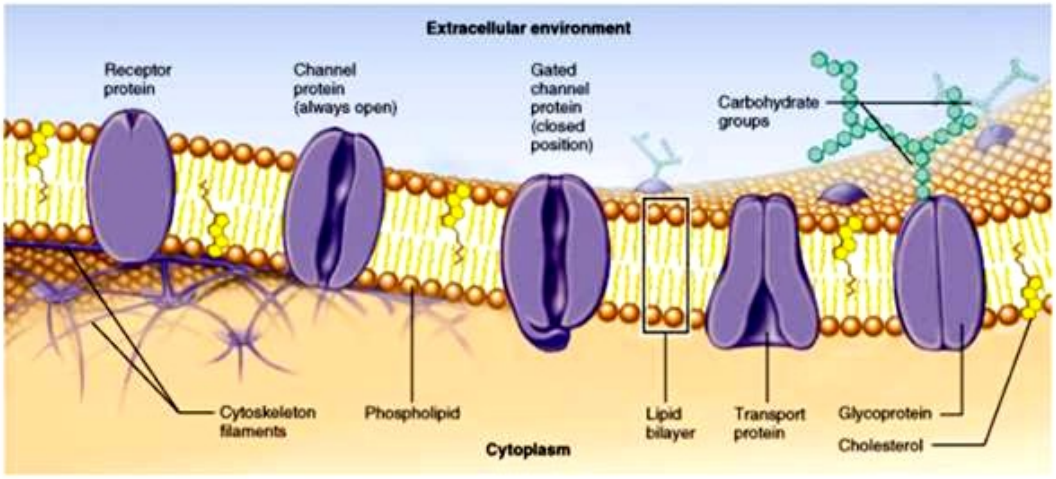

Figure 2.6 : Illustration of complex anatomy of cell membrane. Adapted from (http://bealbio.wikispaces.com/Cell+Membrane /, 2013).

Membranes commonly contain a number of proteins, phospholipids and glycolipids with various head groups, number of chains and chain lengths. In spite of the various complexities, membranes can be generalized to have the significant property that they exist as thin bilayer membranes. As the biological lipids tend to self assemble, these structures are not fixed and are part of a very dynamic system. Each of these three major lipid classes has a polar (hydrophilic) and a nonpolar (hydrophobic) part. Typically, the polar part is rather compact and the nonpolar part is more lengthened, so they are often referred to as the "head" and the "tails" of the lipid molecule, respectively (refer Figure 2.7).

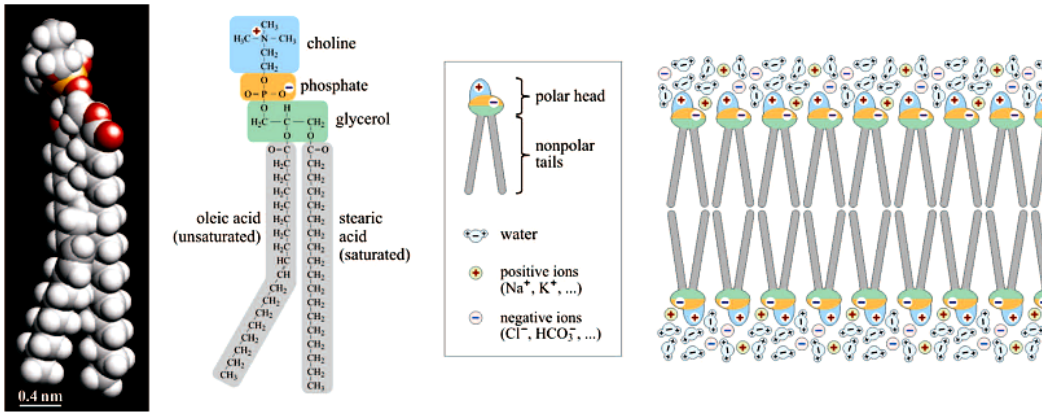

Figure 2.7 Left: space-filling model and structural formula of the SOPC (1-stearoyl-2-oleoyl-phosphatidylcholine) molecule, a typical membrane lipid. Right: a bilayer of such lipids in an aqueous electrolyte solution. Adapted from (Kotnik, Kramar *et al.* 2012).

Since this structure, lipids in aqueous solutions spontaneously form a bilayer, such as a sheet like structure two molecules thick, with the nonpolar tails oriented inward and the polar heads pointing outward, contacting the water and dissolved ions surrounding the bilayer (Figure 2.7, right). A bilayer lipid has a very low electric conductivity and is thus closed for ion transport. The membrane thickness is about 5 nm, thus the membrane capacitance is very high, and the breakdown potential is low. However membranes will change their lipid composition in response to external

stimuli. This will create stress on the membrane, which may form a pore of a non-bilayer structure.

Under certain conditions, such as sufficiently high temperature, surface tension, or both, this permeation can be attributed to the formation and rapid resealing of very small aqueous pores in the lipid bilayer, with radii below a nanometer and lifetimes below a nanosecond; they form and reseal because of thermal and mechanical fluctuations. This explanation is consistent with theory (Litster 1975), (Lawaczeck 1988), and has been corroborated by molecular dynamics (MD) simulations (Shillcock and Seifert 1998; Leontiadou, Mark *et al.* 2004; Gurtovenko, Anwar *et al.* 2010). The pores can form without external electric field acting on the membrane, but they are inherently unstable.

2.4.2. Induced Transmembrane Potential

Once, biological cell was exposed to an electric field, a local distortion of the field in the cell and its vicinity takes place. Due to the low membrane conductivity, the field is concentrated in the cell membrane, where it is several orders of magnitude larger than in the cytoplasm and the extracellular region. This result in an induced transmembrane potential V_m, that is stochastic. This transmembrane potential superimposes to the membrane rest potential (Weaver 1993). When an isolated spherical cell is exposed to a DC homogeneous electric field, the voltage induced on the cell membrane is determined by solving Laplace's equation. For the first approximation, the cell membrane can also be treated as initially nonconductive. Under these assumptions, the transmembrane potential is given by the Schwan's equation, see equation (2.1) (Schwan 1957). Schwan's equation implies that the transmembrane potential varies proportionally to the cosine of the angle and the maximum potential is induced at the points where the electric field is perpendicular to the membrane, namely at $\theta = 0^o$ and $\theta = 180^o$, the points referred to as the `poles' of the cell. The formula describes the static situation, and can safely be applied to yield the steady-state value of the induced transmembrane potential.

$$V_m = \frac{3}{2} EaCos\,\theta \tag{2.1}$$

In the equation (2.1) E is the applied external electric field strength in volts per centimeter, a is the radius of the cell, and θ is the angle between the field line and a normal from the centre of the sphere to a point of interest on the cell membrane. The 1V is a critical transmembrane potential built for electroporation occurs. This situation is called 'electrical breakdown' by Sale and Hamilton (Sale and Hamilton 1967).

However this situation clearly shows that the field-induced increase in permeability is temporary even long-lived compared to the field. In accordance with the terms `electropermeabilization 'used to describe the permeability changes introduced by electric impulses in vesicular membrane (Neumann and Rosenheck 1972). This was later proved by Rosenheck et al. (Rosenheck, Lindner *et al.* 1975) that the change is temporary due to the electric field, while changes in membrane resistance has been associated with dielectric breakdown (Zimmerman., Pilwat. *et al.* 1974).

Consequent studies showed that the cell membranes of pulse treated cells were permeable to molecules of a size smaller than a certain limit, suggesting the creation of a porous membrane structure (Neumann and Rosenheck 1972; Zimmerman., Pilwat. *et al.* 1974). Electroporation conditions occur as a result of dielectric breakdown of the cell membrane appears to generate `holes' or 'pores' that can pass through the material (Hofmann and Evans 1986). It was also found that under appropriate conditions, the cells could recover, which implied that these electropores were resealable and could be induced without permanent damage to the cell (Benz, Beckers *et al.* 1979), and the cytoplasmic macromolecular contents could be retained (Kinosita and Tsong 1977). Since then, a number of research groups have studied mechanisms of pore formation and detailed characteristics of the cell membranes modified by electric fields (Abidor, Arakelyan *et al.* 1979; Chernomordik, Sukharev *et al.* 1983; Schwister and Deuticke 1985; Glaser, Leikin *et al.* 1988).

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